RESEARCH

Response of Low Seed Phytic Acid Soybeans to Increases in External Phosphorus Supply

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ABSTRACT

Commercialization of soybean [*Glycine max* (L.) Merr.] varieties with low seed phytic acid will depend on the stability of the trait when grown in soils with a wide range of P availabilities and on the impact of altered P composition on seed protein and oil concentrations. Impacts of deficient (0.05 mmol L^{-1}) to excessive (0.9 to 1.2 mmol L^{-1}) levels of external P on seed P composition of normal and low phytic acid lines and of altered seed P composition on seed protein and oil synthesis were evaluated. Soybean lines homozygous recessive (*pha*/*pha*) at one of two loci with genes that condition the low seed phytic acid trait had the same greater-than-threefold increase in phytic acid in response to increasing external P as their normal phytic acid parent, 'AGS Prichard-RR' (*Pha*/*Pha*). This supports the conclusion from previous inheritance studies that the low seed phytic acid trait in CX1834-1-2 is controlled by epistatic interaction between two independent recessive genes. The seed phytic acid concentration in the low phytic acid line G03PHY-443 (derived from CX1834-1-2) was <2 g phytic acid P kg^{-1} dry wt. when grown under deficient to excessive external P. As the P supply increased, seed inorganic P concentrations for this line increased from 0.8 to 4.0 g kg^{-1} dry wt., compared to an increase of 0.2 to 0.6 g kg^{-1} dry wt. for the normal phytic acid lines. Seed protein and oil concentrations did not differ significantly between normal and low phytic acid lines. These results support continued development of varieties with low seed phytic acid and high yields.

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Abbreviations: HPLC, highperformance liquid chromatography; LG, linkage group; SEPEL, Southeastern Plant Environment Laboratories; SSR, simple sequence repeat.

Phytic acid (inositol 1,2,3,4,5,6-hexa*kis*phosphate) is the most abundant form of P in seeds of many crop plants [maize, *Zea mays* L.; rice, *Oryza sativa* L.; wheat, *Triticum aestivum* L.; barley, *Hordeum vulgare* L.; and soybean, *Glycine max* (L.) Merr.]. Phytic acid contains 65 to 80% of the total P in mature soybean seed (Raboy and Dickinson, 1993) and in the cereal grains (Raboy, 1997). Although soybean meal is a major source of protein in rations for swine and poultry, these monogastric animals lack sufficient phytase enzyme to cleave most of the P from phytate (Ertl et al., 1998). For maize, only 10 to 20% of P is available to swine and poultry (Cromwell et al., 1993). Poultry and swine accounted for 18% of the total animal waste in the USA in 1991, yet these animals accounted for one-third of the total P excreted in animal waste (Cromwell and Coffey, 1991). Phytic acid also has antinutritional properties, as it chelates mineral nutrients such as Cu, Zn, Mn, Fe, and Ca, thus reducing their bioavailability (Ertl et al., 1998).

Supplementing rations with inorganic P or bone meal to meet animal P requirements has been used to overcome the P availability problem, but this practice increases P excretion in the manure (Cromwell and Coffey, 1991). An alternative approach that is more

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environmentally friendly has been to add phytase to feed rations. Phytase additions to feed have increased P availability of corn from 15 to 43% in swine (Cromwell et al., 1993) and reduced P excretion by broilers as much as 24%.

Phytic acid accumulation in soybean seed begins in early embryogenesis and is linear throughout most of seed development (Raboy and Dickinson, 1987). External P availability influences phytic acid P concentrations in soybean seed (Raboy and Dickinson, 1993). For example, phytic acid P concentrations in seed of six soybean cultivars increased an average of four-fold (1.6 to 6.6 g kg^{-1}) when the external P supply to plants was increased from 2 to 50 mg L^{-1} (Raboy and Dickinson, 1993). Increases in phytic acid P accounted for almost all of the increase in total seed P, as inorganic P and other nonphytic acid P were essentially constant across the range of external P concentrations.

Since the mid-1990s genetic and breeding approaches have been used to reduce phytic acid P concentrations and increase bioavailability of P in seeds of grain crops (Larson and Raboy, 1999; Raboy et al., 2000; Shi et al., 2003; Wilcox et al., 2000) used in animal rations, and to ameliorate problems with P excretion in animal manure (Ertl et al., 1998). Genotypes with low seed phytic acid P concentrations have been developed in maize (Larson and Raboy, 1999; Raboy et al., 2000; Shi et al., 2003), rice (Larson et al., 2000), barley (Larson et al., 1998; Dorsch et al., 2003), wheat (Guttieri et al., 2004), and soybean (Wilcox et al., 2000).

Chemical mutagenesis has been used to obtain mutants with alterations in different steps of the phytic acid biosynthetic pathway and several classes of phenotypes have been described. Mutations which cause reciprocal effects on seed phytic acid and inorganic P concentrations in seed, with no accompanying change in the total P concentration have been designated as low phytic acid 1 (*lpa1*) mutations (Raboy et al., 2000). Two soybean mutants that fall in the *lpa1* class have been identified (Hitz et al., 2002; Wilcox et al., 2000). Other mutations which cause a decrease in phytic acid P concentration and an accumulation of inositol phosphate intermediates have been designated as *lpa2* mutations (Raboy et al., 2000; Shi et al., 2003). Recently, a maize mutation that decreases phytic acid P concentration in grain by 66% and causes an accumulation of inositol has been reported (Shi et al., 2005). This mutation was designated as *lpa3*. The *lpa1*, *lpa2*, and *lpa3* mutations can reduce seed phytic acid concentrations by 30% to >90% (Hitz et al., 2002; Larson et al., 1998; Raboy et al., 2000; Shi et al., 2003, 2005).

Wilcox et al. (2000) developed a soybean mutant with seed phytic acid P concentrations that are 80% lower than normal. Genes from this material have been crossed into germplasm adapted to several regions of the United States. Inheritance studies have shown that the low seed phytic acid phenotype in CX1834-1–derived lines descended from the soybean mutant of Wilcox et al. (2000) is conditioned by two independently segregating recessive genes designated as *pha1* and *pha2* (Oltmans et al., 2004). The enzymatic steps in the phytic acid biosynthetic pathway that have been altered in these mutants to generate the low phytic acid phenotype have not been identified, but loci with recessive alleles conditioning low phytic acid in CX1834-1 have been mapped to molecular linkage groups (LGs) N and L (Walker et al., 2006). These genes presumably correspond to *pha1* and *pha2*, and heterozygotes have phytic acid levels (based on inorganic P levels) that are generally similar to those of the wild-type parents (Walker et al., 2006). Data from markers closely linked to the phytic acid loci on LGs L and N have been used to identify backcross-derived lines homozygous for the low phytic acid allele at either or both of the loci (R. Boerma, personal communication, 2006).

The response of seed P composition of low phytic acid lines to increased external P availability has not been reported. Since phytic acid synthesis is impaired in these lines, we hypothesized that increased P availability would cause accumulation of high inorganic P concentrations and have little impact on the phytic acid P concentrations in seed of low phytic acid lines. If phosphorylation–dephosphorylation of enzymes such as cytosolic pyruvate kinase regulate the flow of C between protein and oil synthesis (Sebastia et al., 2005), concentrations of these constituents may be altered by the accumulation of high inorganic P concentrations in seed of low phytic acid lines.

Soils in soybean production areas tend to have high levels of available P, especially those that have received manure applications for a long period of time (Sims et al., 2000). For the low phytic acid trait to be useful in soybean production systems, it must be stable over a range of available soil P levels. The objectives of this study were to assess (i) the stability of the low seed phytic acid trait across levels of external P supply ranging from deficient to excessive, and (ii) the impact of the low seed phytic acid trait on seed protein and oil concentrations.

MATERIALS AND METHODS

Plant Materials and Genotypes

Experiment 1

A P nutrition experiment was conducted with a pair of low (G03PHY-443) and normal ('AGS Prichard-RR', referred to hereafter as Prichard-RR) seed phytic acid lines of soybean developed by Roger Boerma at the University of Georgia. Prichard-RR is a glyphosate-tolerant version of the Maturity Group VII cultivar Prichard, which has a normal seed phytic acid content (Boerma et al., 2001). Line G03PHY-443 was derived from a cross between Prichard-RR and CX1834-1-2, a low phytic acid line obtained from J.R. Wilcox (USDA-ARS and Purdue University). Both low phytic acid alleles from CX1834-1-2 (Wilcox et al., 2000) were backcrossed into Prichard-RR using marker-assisted selection.

G03PHY-443 is a BC_4F_2–derived line that is homozygous for low phytic acid alleles at loci on LGs L and N (i.e., its genotype is (*pha1pha1pha2pha2*). Outside the regions surrounding these loci, the genome should be approximately 97% Prichard-RR.

Experiment 2

Unique genetic material from the breeding program of Roger Boerma was used to evaluate the impact of individual recessive genes conditioning the low phytic acid trait on differences in seed P composition in response to different external P concentrations. The four genotypes (Prichard-RR, G03PHY-443, LG L, and LG N) used in this experiment all had a predominantly Prichard (Boerma et al., 2001) genetic background. Individual low phytic acid alleles from CX1834-1-2 (Wilcox et al., 2000) were backcrossed into Prichard-RR using marker-assisted selection. The LG L and LG N lines are derived from the same BC_4F_1 parent as GO3PHY-443 but carry low phytic acid alleles at only one of the two mapped phytic acid loci. The LG L line has the *pha2/pha2* genotype at the phytic acid locus near Satt561 on LG L, but is homozygous for the *Pha1* alleles of the recurrent parent at the other phytic acid locus near Satt237 on LG N (Walker et al., 2006). In contrast, the LG N line has the *pha1/pha1* genotype at the phytic acid locus on LG N but is homozygous for the Prichard-RR *Pha2* alleles at the LG L locus.

Plants used in this experiment were genotyped at simple sequence repeat (SSR) markers on LGs L and N to confirm the presence of the expected parental allele at each of the two phytic acid loci. Immature leaves (1–2 cm long) were sampled 25 d after planting and lyophilized. DNA was obtained from this material using a CTAB-based extraction protocol (Keim et al., 1988) and polymerase chain reaction protocols followed those described by Walker et al. (2006). Samples of Prichard-RR and CX1834-1-2 DNA were included as controls. Satt339 and Satt237, which flank the estimated position of the phytic acid locus on LG N, were used to determine the genotype at that locus (Walker et al., 2006). Satt166 and Satt113, which are 4 to 5 cM to one side of the estimated location of the LG L phytic acid locus, respectively, had to be used because SSR markers closer to the locus were monomorphic and therefore uninformative.

Plant Culture and Environmental Conditions

Experiment 1

Plants were grown in controlled environment chambers (Environmental Growth Chambers Co., Chagrin, OH, as modified by the Southeastern Plant Environment Laboratories [SEPEL]) with 800 μmol m^{-2} s^{-1} of photosynthetically active radiation and 400 μL L^{-1} CO_2. A day/night temperature of 26/22°C (±0.50°C of set point in day and ±0.25°C of set point at night) was used throughout the experimental period. A 9-h photosynthetic period and a 15-h dark period interrupted with photomorphogenic irradiance for 3 h (to prevent flowering) were used daily until the second trifoliolate leaf began to unroll. At this stage, the night interruption was discontinued to induce flowering.

Three seeds of the soybean lines described above were planted into thoroughly moistened peatlite–gravel mix (SUNGRO Horticulture, Bellevue, WA) in 25-cm (6-L) pots and placed into the controlled environment chambers. After emergence, pots were thinned to one healthy seedling. Plants were supplied nutrient solution (Thomas and Downs, 1991) modified to contain deficient (0.1 mmol L^{-1}), sufficient (0.5 mmol L^{-1}), or excessive (1.2 mmol L^{-1}) P and 15.0 mmol L^{-1} N. Each genotype × P level treatment combination was replicated three times. From emergence until 35 d after planting, pots were flushed at 0900 and 1400 h each day with deionized water, with the addition of 0.5 L of appropriate nutrient solution after the 1400-h flush. From 35 to 85 d after planting, 0.5 L of the appropriate nutrient solution was applied after both flushes with deionized water. From 85 d after planting until harvest, nutrient solution application was discontinued to enhance maturation.

At harvest maturity, pod number, seed number, and seed, leaf, and stem dry mass data were collected. A subsample of seed from each plant was dried to constant weight at 60°C, and was ground sufficiently to pass through a 1-mm screen before being used for measurement of inorganic P, phytic acid P, protein and oil concentrations, and oil composition.

Experiment 2

Except for indicated changes, culture conditions were the same as for Experiment 1. Plants were grown in large walk-in controlled environment chambers with 600 μmol m^{-2} s^{-1} of photosynthetically active radiation. This change was needed because chambers used in Experiment 1 were in use by others and would not have been available for 4 mo. Plants were supplied nutrient solution as described in the North Carolina University Phytotron Procedural Manual (Thomas and Downs, 1991). The nutrient solutions were modified to contain deficient (0.05 mmol L^{-1}), sufficient (0.50 mmol L^{-1}), or excessive (0.90 mmol L^{-1}) P levels. The deficient P and excessive P levels were decreased to 0.05 mmol L^{-1} and 0.90 mmol L^{-1}, respectively, because less growth potential was expected at the 25% lower light intensity used in this experiment and the 0.1 mmol L^{-1} P treatment used in Experiment 1 did not decrease seed yield significantly (Tables 1 and 2). Genotype × P level treatment combinations were replicated three times.

Chemical Analyses

All samples were dried to a constant weight at 60°C before weighing. Phytic acid P content in soybean seed was determined as previously described (Kwanyuen and Burton, 2005) with some modifications. Unless otherwise stated, all procedures were performed at room temperature. Soybean seed samples were ground in a ZM 100 centrifugal grinding mill (Retsch, Haan, Germany) equipped with a 24-tooth rotor and 1.0-mm stainless steel ring sieve with the motor speed set at 15,000 rpm. This setting produced ground samples with a uniform particle size of <0.5 mm. For convenience, extractions were performed in 20-mL vials with 0.5 mol L^{-1} HCl in a ratio of 1:20 (w/v) for 1 h with stirring. Samples (0.5 g) were extracted with 10 mL of 0.5 mol L^{-1} HCl. Aliquots (2 mL) of crude extract from each sample were centrifuged at 18,000 *g* for 10 min in a microcentrifuge. A 1-mL aliquot of supernatant containing phytic acid was then filtered through a 13-mm/0.22-μm syringe filter. Filtered samples could be stored at 4°C for as long as 48 h before high-performance liquid chromatography (HPLC) analysis because the strongly acidic (0.5 mol L^{-1} HCl) extractant and the low temperature were not favorable for phytase activity. Optimum pH ranges from 4.0 to 7.5 and optimum temperature

ranges from 35 to 70°C for phytases from different organisms (Phillippy, 2003).

Chromatography was performed on a binary HPLC system with a 4 by 250 mm IonPac AS7 analytical column (Dionex Corp., Sunnyvale, CA) equipped with a 4 by 50 mm IonPac AG7 guard column. Elution of phytic acid was achieved with a 15-min linear gradient of 0.01 mol L^{-1} 1-methylpiperazine (pH 4.0) to 0.5 mol L^{-1} $NaNO_3$ in 0.01 mol L^{-1} 1-methylpiperazine (pH 4.0) at a flow rate of 1 mL min^{-1}. Wade's color reagent (Wade and Morgan, 1955) consisting of 0.015% (w/v) $FeCl_3$ and 0.15% (w/v) 5-sulfosalicylic acid (also at flow rate of 1 mL min^{-1}) and phytic acid that eluted from the column were mixed in a mixing tee, with inline check valves installed before the mixing tee to prevent back flow. The post-column reaction was allowed to take place in a 250-μL sample loop at the combined flow rate of 2 mL min^{-1}. The absorbance was monitored at 500 nm while the detector signals and/or phytic acid peaks were processed and integrated by the chromatographic data acquisition system.

Inorganic P in seed was determined by the microtitre plate assay method described by Larson et al. (2000). This involved extraction of 100 mg of dry seed, ground to pass through a 1-mm screen, with 3.0 mL of 12.5% w/v trichloroacetic acid containing 25 mmol L^{-1} $MgCl_2$. Aliquots (10 μL) of extracts were diluted with 90 μL of deionized water and reacted with 100 μL of Chen's reagent (1 vol. 0.02 mol L^{-1} ammonium molybdate, 1 vol. 10% w/v ascorbic acid, 1 vol. 3.0 mol L^{-1} sulfuric acid, and 2 vol. distilled water) (Chen et al., 1956) in wells of microtitre plates. Two standard curves for inorganic P (0–1.5 μg per well) were established on each plate by adding appropriate volumes of 1.0 mmol L^{-1} K_2HPO_4 to different wells along with the extractant and deionized water. One hour after adding Chen's reagent, absorbance at 882 nm was determined for each well using a microplate reader (Model MQX200, BIO-TEK Instruments, Winooski, VT). The absorption maximum for the phospho-molybdenum blue complex that forms on addition of Chen's reagent is 882 nm (Murphy and Riley, 1962). Absorbance readings for samples were corrected by subtracting absorbance of reagent blanks.

Total P concentration in seeds and leaves was measured with inductively coupled plasma emission spectroscopy (Novozamsky et al., 1986). Total protein was measured by the Dumas reductive combustion method coupled with thermal conductivity detection (AOCS, 1995a). Total oil concentration was determined with pulsed NMR on whole seeds (AOCS, 1995b). Fatty acid composition of oil was measured by the method of Wilson et al. (2001).

Statistics

In both experiments, genotype × P treatment combinations were replicated three times and arranged in a randomized block design (two blocks in one chamber and one block in a second chamber set for the same environmental conditions). The SAS GLM procedure (SAS Institute, 1999) was used for the statistical analysis. Appropriate Fisher Protected LSD values for dependent variables were calculated for comparison of treatment means when either main effects or genotype × P treatment interaction effects were significant at the 0.05 probability level.

Table 1. Significant differences for genotype and P level main effects and genotype (G) × P level interactions on seed attributes measured in Experiment 1.

Trait	Genotype	P level	G × P level	Coefficient variation
				%
Seed mass, g plant^{-1}	*	NS†	NS	14.9
Seed number plant^{-1}	*	NS	NS	12.2
Total P accumulation in seed, mg plant $^{-1}$	*	**	NS	19.9
Total P conc., g kg^{-1} dry wt.	NS	**	*	3.2
Phytic acid P, g kg^{-1} dry wt.	**	**	**	9.6
Phosphate P, g kg^{-1} dry wt.	**	**	**	11.5
Cellular P, g kg^{-1} dry wt.	**	**	NS	6.0
Protein, g kg^{-1} dry wt.	**	**	NS	1.3
Total oil, g kg^{-1} dry wt.	NS	*	*	1.4
Palmitic acid, weight %	**	**	**	1.7
Stearic acid, weight %	*	**	**	5.3
Oleic acid, weight %	NS	**	**	3.9
Linoleic acid, weight %	**	**	**	1.2
Linolenic acid, weight %	**	**	**	8.6

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

†NS, nonsignificant.

Table 2. Influence of genotype and P supply on seed mass and total P accumulation in seed (Experiment 1). Since genotype × P level interactions were not significant, only P treatment means and genotypic means and associated Fisher protected $LSD_{0.05}$ values are presented.

Nutrient solution P conc.	Genotype	Seed mass	Seed number	Total P accumulation in seed
mmol L^{-1}		g plant^{-1}	plant^{-1}	mg plant^{-1}
		P treatment means		
0.1		49.7	241	161
0.5		50.3	266	342
1.2		42.4	237	339
$LSD_{0.05}$		NS†	NS	82
		Genotypic means		
	G03PHY-443	42.3	229	245
	Prichard RR	52.6	266	317
	$LSD_{0.05}$	8.3	35	50

†NS, nonsignificant.

RESULTS AND DISCUSSION

Two of five P nutrition experiments that we conducted with low and normal seed phytic acid genotypes are presented in this report. Response of seed P composition to increasing P supply for the three unpublished experiments was the same as for the two experiments presented in this report. These genotypes (CX1834-A-1-1, normal and CX1834-A-1-4, low) were early selections from Dr. Wilcox's mutagenesis program (Wilcox et al., 2000) and were not as genetically

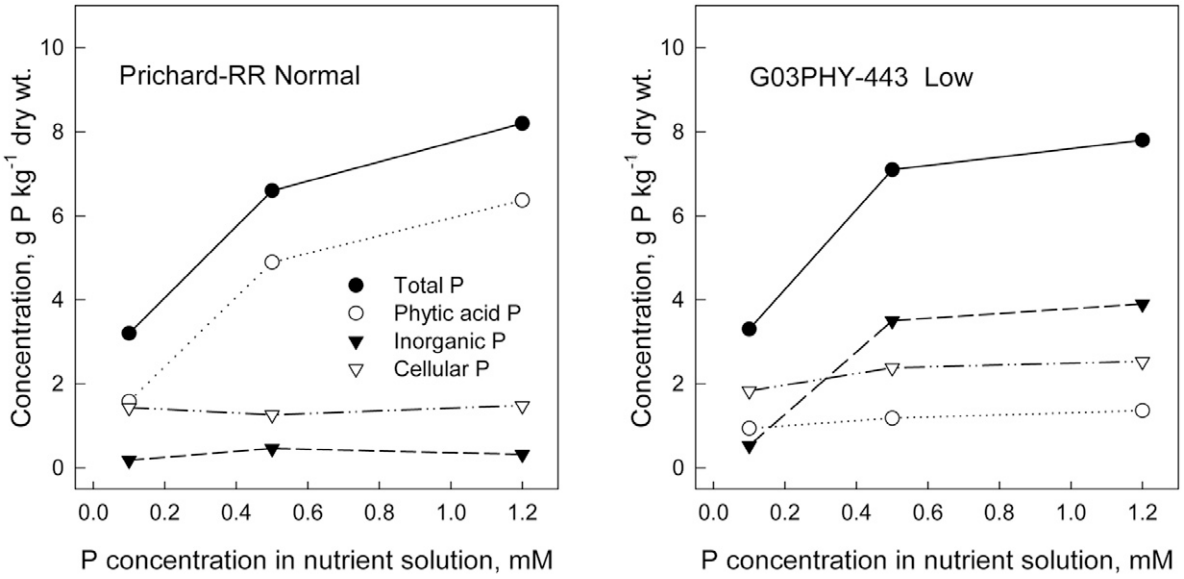

Figure 1. Influence of P supply on seed P composition of normal and low phytic acid soybeans (Experiment 1). $LSD_{0.05}$ values for comparison of any two treatment means are 0.42, 0.32, and 0.37 for phytic acid P, inorganic P, and total P concentrations, respectively, as P level × genotype interactions were significant. Genotype and P level main effects on cellular P concentration were also significant at the 0.0001 probability level.

uniform as the genotypes used in experiments reported here. For this reason we chose to present two experiments with the low seed phytic acid genotype (G03PHY-443) because it was derived from four backcrosses with Prichard-RR and as a result has 97% of the Prichard-RR genome. Genotypes in the same genetic background that are homozygous recessive for one of two genes that condition the low seed phytic acid trait were also included in Experiment 2.

Seed Production

Experiment 1

A summary of significant differences among genotype and P treatment main effects and genotype × P treatment interactions for plant and seed attributes is presented in Table 1. External P supply had no significant effect on total seed mass. However, the low phytic acid line (G03PHY-443) had 20% lower seed mass than the normal phytic acid line (Table 2). Differences in seed number accounted for most of the difference in seed mass between the low and normal phytic acid lines (Table 2). Phosphorus treatments had significant effects on total P accumulated in the seed (Table 1 and 2). Total P accumulation in the seed (mg seed P plant $^{-1}$) of both lines was essentially equal at the 0.5 and 1.2 mmol L^{-1} external P (Table 2). Most of the increase in total seed P accumulation was the result of large increases in seed P concentration (Fig. 1). These results indicate that the highest external P concentration was excessive with respect to production of total seed mass per plant, but 0.1 mmol L^{-1} P was not deficient.

Experiment 2

Statistical information is summarized in Table 3. Total seed mass of the low phytic acid line G03PHY-443 was 33% lower than that of the normal phytic acid lines (Table 4), however this difference was not significant at the 0.05 level because of relatively high variability (Table 3). Total seed mass increased significantly between the 0.05 and 0.50 mmol L^{-1} P levels. While seed mass was statistically similar for the 0.5 and 0.9 mmol L^{-1} P treatments, seed mass tended to be lower in the 0.9 mmol L^{-1} P treatment (Table 4). Total seed mass response confirms that the external P levels ranged from deficient to excessive as 0.5 and 0.9 mmol L^{-1} P treatments produced the same seed yield. Genotype and P treatment had significant effects on total P accumulation in the seed (Table 4). Total seed P reached a maximum level at 0.50 mmol L^{-1} P, and the normal phytic acid lines accumulated 44% more total P in seed (0.07 probability level) than the low phytic acid line (Table 4).

Phosphorus Composition of Seed and Leaves

Experiment 1

Phytic acid P increased from 1.6 to 6.4 g kg^{-1} dry wt. in seed of the normal phytic acid line (Prichard-RR), and from 0.9 to 1.4 g kg^{-1} dry wt. in seed of the low phytic acid line (G03PHY-443) as P supply increased from deficient to excessive levels (Fig. 1). In contrast, the inorganic P concentration in seed of the normal phytic acid line was relatively stable, increasing from 0.2 to 0.3 g kg^{-1} dry wt. as P supply increased. Conversely, the inorganic P concentration in seed of the low phytic acid line increased from 0.5 to 3.1 g kg^{-1} dry wt. as the P supply was increased from deficient to above sufficient levels (Fig. 1). These responses of seed phytic acid P and inorganic P concentrations to external P supply are the same as those obtained for a pair

of low (CX1834A-1-4) and normal (CX1834A-1-1) seed phytic acid lines developed by Dr. J.R. Wilcox at Purdue University (Israel, unpublished observations, 2005).

The cellular P fraction is derived by subtracting inorganic P and phytic acid P concentrations from the total P concentration in seed. This fraction contains other soluble P metabolites, RNA and DNA. The concentration of cellular P was consistently higher in seed of the low phytic acid line than in normal phytic acid lines at all levels of P supply (Fig. 1). The cellular P concentration in seed of the low phytic acid line increased 38% as the P supply was increased from 0.1 to 1.2 mmol L^{-1} (Fig. 1).

When the external P concentration was increased from sufficient (0.5 mmol L^{-1}) to excessive (1.2 mmol L^{-1}) levels, total P concentration in seed of the normal phytic acid line increased 14% while the total P concentration in leaves increased 400%. Similarly, the total P concentration in seed of the low phytic acid line increased about 15% between sufficient and excessive external P concentrations while total P concentrations in leaves increased 360% (Fig. 2). The inorganic P concentration in seed of the low phytic acid line increased 41% between sufficient and excessive external P concentrations (Fig. 1). These results show that, while P uptake may not be down-regulated at excessive external P concentrations, the additional P accumulated in vegetative tissues is not translocated to the developing seed. This indicates physiological limits on the concentration to which total P, phytic acid P, and inorganic P can accumulate in seed when external P is in excess of crop requirements. Thus, when low phytic acid lines are grown on soils with high soil test P, concentrations of P constituents should not increase to levels that impair physiological functions of the seed.

Experiment 2

The normal phytic acid line Prichard-RR and the genotypes with homozygous recessive (*pha/pha*) alleles at one of two known loci governing the seed phytic acid levels (LG L and LG N), exhibited almost identical increases in seed phytic acid P concentration (2.0 to 7.0 g kg^{-1} dry wt) in response to increased external P supply (Fig. 3). The inorganic P concentrations in seed of these genotypes were low (0.2–0.6 g kg^{-1} dry wt) and showed minimal response to increased P supply in comparison to the low phytic acid line (Fig. 3). In contrast, seeds of the G03PHY-443 line, which is homozygous recessive (*pha/pha)* at both loci controlling the seed phytic acid levels, had 2.0 g phytic acid P kg^{-1} dry wt, and this did not increase as the external P supply was increased. The inorganic P concentration in seed of this line increased five-fold as external P supply was increased from deficient to excessive levels (Fig. 3). Since the scale used in Fig. 3 masks the effects of P supply on the seed inorganic P concentration in lines with normal phytic acid concentrations, these data were plotted using a more sensitive scale (Fig. 4). At the 0.9 mmol L^{-1} external P level, seed inorganic P concentration was significantly greater ($LSD_{0.05}$ = 0.22 g kg^{-1} dry wt) for LG N, which has *pha1/pha1* alleles at a locus on LG N and *Pha2/Pha2* alleles at a locus on LG L, than for LG L, which has *pha2/pha2* alleles at a locus on LG L and *Pha1/Pha1* alleles at a locus on LG N. This is consistent with molecular marker evidence that the locus on LG N has a greater effect on inorganic P levels than the locus on LG L (Walker et al., 2006).

Table 3. Significant differences for genotype and P level main effects and genotype (G) × P level interactions on seed attributes measured in Experiment 2.

Trait	Genotype	P level	G × P level	Coefficient variation
				%
Seed mass, g $plant^{-1}$	NS†	**	NS	29.7
Seed number $plant^{-1}$	*	**	NS	27.3
Total P accumulation in seed, mg $plant^{-1}$	NS	**	NS	30.8
Total P conc., g kg^{-1} dry wt.	*	**	NS	7.6
Phytic acid P, g kg^{-1} dry wt.	**	**	**	7.5
Phosphate P, g kg^{-1} dry wt.	**	**	**	18.7
Cellular P, g kg^{-1} dry wt.	**	NS	NS	17.8
Protein, g kg^{-1} dry wt.	NS	**	*	2.1
Total oil, g kg^{-1} dry wt.	**	**	NS	1.8
Palmitic acid, weight %	**	*	NS	4.0
Stearic acid, weight %	NS	**	**	3.1
Oleic acid, weight %	**	NS	NS	3.9
Linoleic acid, weight %	**	**	NS	1.9
Linolenic acid, weight %	**	**	**	3.3

*Significant at 0.05 level.

**Significant at the 0.01 level.

†NS, nonsignificant.

Table 4. Influence of genotype and external P supply on seed mass and total P accumulation in seed (Experiment 2). Since genotype × P level interactions were not significant, only P treatment means and genotypic means and associated Fisher protected $LSD_{0.05}$ values are presented.

Nutrient solution P conc.	Genotype†	Seed yield	Seed number	Total P accumulation in seed
	mmol L^{-1}	g $plant^{-1}$	$plant^{-1}$	mg $plant^{-1}$
		P treatment means		
0.05		24.1	122	89
0.50		40.0	205	301
0.90		33.5	200	284
$LSD_{0.05}$		8.5	42	61
		Genotypic means		
	G03PHY-443	24.1	129	169
	Prichard RR	36.4	213	253
	LG L	33.0	168	232
	LG N	36.5	193	245
	$LSD_{0.05}$	NS	48	NS

†LG L, linkage group L; LG N, linkage group N.

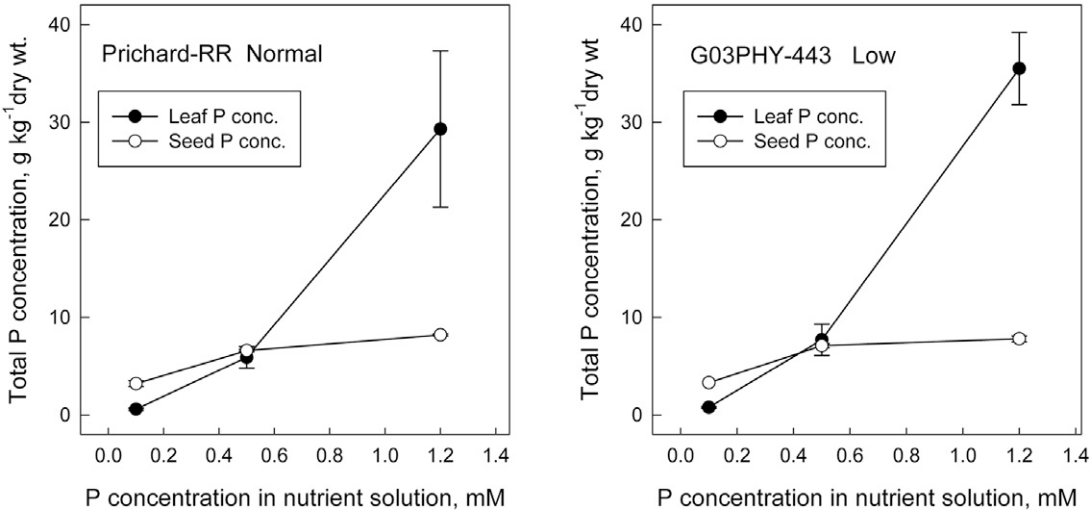

Figure 2. Impact of P supply on total P concentrations in leaves and seed of soybean genotypes with normal and low seed phytic acid P concentrations (Experiment 1). Bars represent standard deviations.

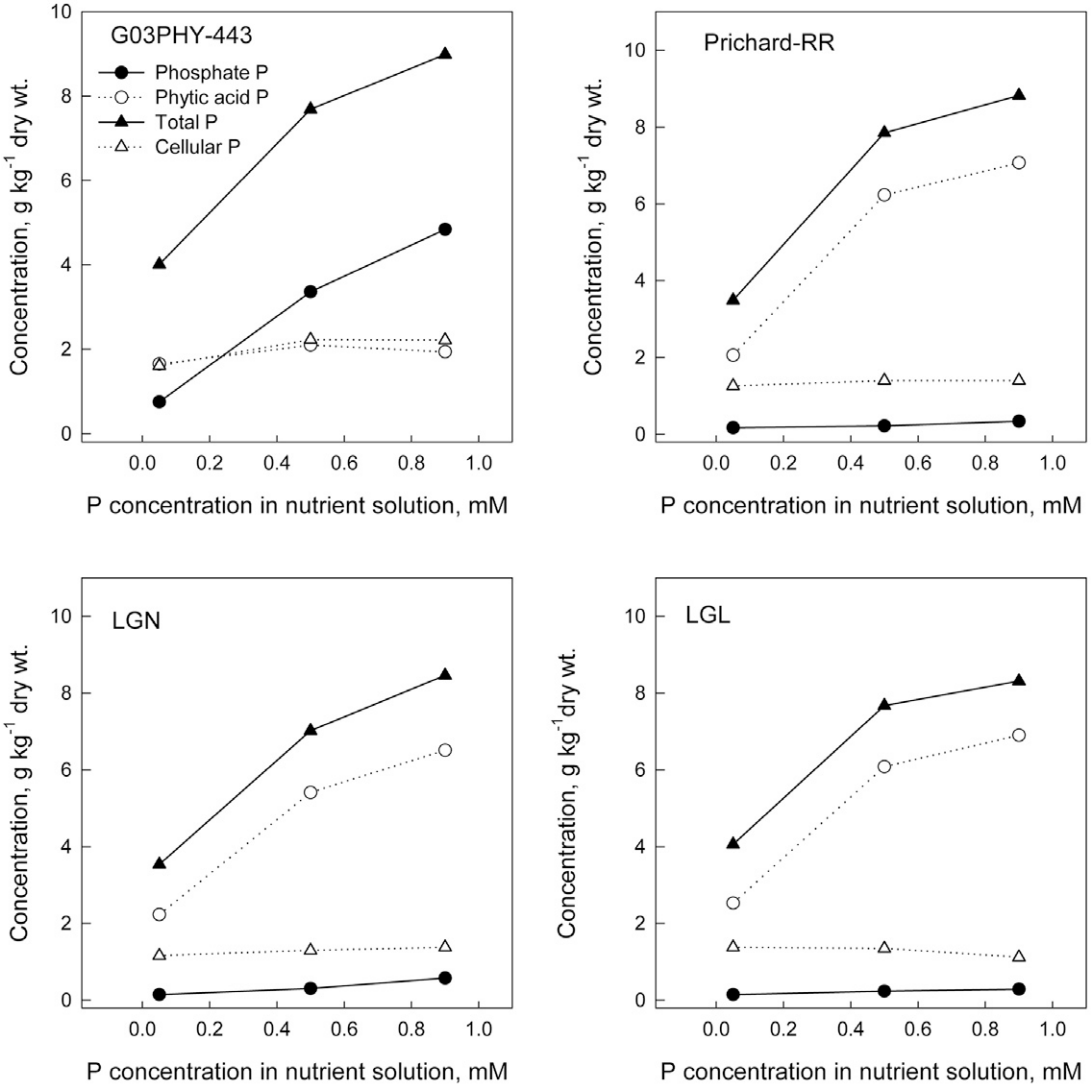

Figure 3. Impact of external P supply on seed P composition of a soybean genotype with no low phytic acid alleles (Prichard RR) and of genotypes with either low phytic acid alleles on linkage group L (LG L), or low phytic acid alleles on linkage group N (LG N) or low phytic acid alleles on both LG L and LG N (G03PHY-443) (Experiment 2). $LSD_{0.05}$ values for comparison of any two treatment means are 0.56 and 0.22 for phytic acid P and inorganic P concentrations, respectively, as the P level × genotype interactions were significant at the 0.05 probability level. The genotype main effect for cellular P concentration and the P level main effect for total P concentration were significant at the 0.0001 probability level.

These results clearly demonstrate that *pha/pha* alleles at both loci are obligatory for prevention of increases in seed phytic acid concentration in response to increased P supply. They also provide additional support for conclusions from inheritance and mapping studies that the low phytic acid trait in lines derived from line CX1834-1-2 or its sister line CX1834-1-6 is conditioned by two recessive genes with an epistatic interaction (Fig. 4) (Oltmans et al., 2004; Walker et al., 2006).

The cellular P concentration was 70% higher in seed of G03PHY-443 than in seed of the three normal phytic acid lines at the excessive external P level (Fig. 3). Oltmans et al. (2005) reported that low phytic acid seed had 50% higher cellular P concentrations than normal phytic acid seed when lines were tested in three different field environments. Cellular P levels in the low phytic acid line also increased 38% as external P supply increased from deficient to excessive levels (Fig. 3). Consistently greater cellular P concentrations in seed of the low phytic acid line than of normal lines and increased cellular P concentrations in seed of low phytic acid lines at high external P (Fig. 1 and 3) suggest accumulation of metabolites caused by impairment of an early step in the phytic acid biosynthetic pathway. Partially phosphorylated intermediates of inositol were not observed in HPLC chromatograms for low phytic acid seed (unpublished observations). Wilcox et al. (2000) also noted the absence of partially phosphorylated intermediates in seed from the original low phytic acid soybean mutants from which lines used in this study were derived. This indicates that phosphorylation of inositol via inositol-3 kinase or earlier steps in the biosynthetic pathway may be impaired in low phytic acid lines as has been observed in maize (Shi et al., 2005). If the inositol-3 kinase step is impaired, the seed of low phytic acid lines should have higher inositol concentrations than seed of normal phytic acid lines.

Protein and Oil Content

In both experiments, protein concentrations in seeds of low and normal phytic acid lines were similar (44–45%) at the deficient P level and decreased about 5% with increasing P supply (Tables 5 and 6). Genotype had no significant effect on the seed protein concentration (Tables 5 and 6). Hence, changes in protein concentration were associated with P nutrition and not with the phytic acid phenotype of the lines. Total oil concentrations in the seed of the low and normal phytic acid lines were similar and relatively stable between deficient and excessive P levels (Tables 5 and 6). The oil concentrations were marginally higher in Experiment 1 than in Experiment 2 (Tables 5 and 6). Collectively, these results indicate that changes in seed P composition from predominately phytic acid P to predominately inorganic P did not alter the balance between protein and oil synthesis during seed development.

Table 5. Seed protein and oil concentrations of low and normal phytic soybean genotypes in response to P supply (Experiment 1).

Genotype	Nutrient solution P conc.	Total protein	Total oil
	mmol L^{-1}	g kg^{-1} dry wt	
G03PHY-443	0.1	440	210
	0.5	436	199
	1.2	410	202
Prichard RR	0.1	459	203
	0.5	441	203
	1.2	430	207
	$LSD_{0.05}$	NS†	6
		P treatment means	
	0.1	449	206
	0.5	438	201
	1.2	420	204
	$LSD_{0.05}$	8	4
		Genotypic means	
G03PHY-443		429	204
Prichard RR		443	204
$LSD_{0.05}$		7	NS

†NS, nonsignificant.

Table 6. Influence of low phytic acid mutations and P supply on protein and oil composition of soybean seed (Experiment 2).

Genotype†	Nutrient solution P conc.	Total protein	Total oil
	mmol L^{-1}	g kg^{-1} dry wt	
G03PHY-443	0.05	455	190
pha1pha1/pha2pha2	0.50	406	201
	0.90	417	194
Prichard RR	0.05	455	196
Pha1Pha1/Pha2Pha2	0.50	420	202
	0.90	428	203
LG L	0.05	455	197
Pha1Pha1/pha2pha2	0.50	410	213
	0.90	405	211
LG N	0.05	451	199
pha1pha1/Pha2Pha2	0.50	433	206
	0.90	415	209
	$LSD_{0.05}$	20	NS
		P treatment means	
	0.05	454	196
	0.50	417	206
	0.90	416	204
	$LSD_{0.05}$	8	3
		Genotypic means	
G03PHY-443		426	195
Prichard-RR		434	200
LG L		423	207
LG N		433	205
$LSD_{0.05}$		NS‡	4

†LG L, linkage group L; LG N, linkage group N.

‡NS, nonsignificant.

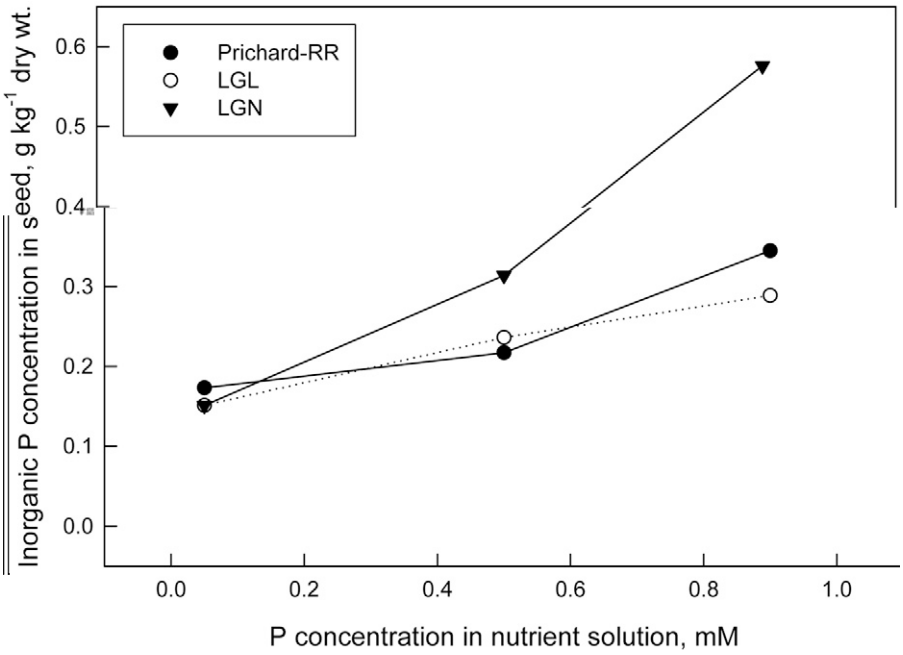

Figure 4. Effect of external P supply on seed inorganic P concentration of a genotype with no low phytic acid alleles (Prichard-RR) compared to response in lines with low phytic acid alleles at a locus on linkage group L (LG L) or at a locus on linkage group N (LG N) (Experiment 2). The $LSD_{0.05}$ for comparison of any two treatment means is 0.22.

Genotype and P treatment had small but statistically significant impacts on fatty acid composition of soybean oil (Tables 7 and 8). Increasing the external P supply increased the oleic acid (18:1) as a percentage of total oil by 30% in Experiment 1 (Table 7). This increase in 18:1 was associated with a similar decrease in linoleic acid (18:2) as a percentage of total oil. However, genotype had no significant effect on the percentage of 18:1 in Experiment 1. These results suggest that increasing the P supply to the developing seed decreased the activity of the desaturase that converts 18:1 to 18:2. In Experiment 2, the external P supply had no influence on the relative amounts of 18:1 and 18:2. The lines with normal levels of phytic acid (Prichard RR, LG L, and LG N) had significantly higher percentages of 18:1 than the low phytic acid line (Table 8). We have no explanation for the inconsistent influence of P supply and genotype on oleic acid content of the oil. Palmitic acid comprised a significantly greater proportion (5–16%) of total oil in seed of G03PHY-443 as compared to the genotypes with normal phytic acid levels (Tables 7 and 8).

A quantitative trait locus associated with seed fatty acid composition, especially oleic acid, maps very close to Satt561 on LG L (R^2 = 0.10) (Hyten et al., 2004; M. Monteros, personal communication, 2006). Selection for the CX1834-1-2 allele at this marker is therefore likely to transfer both the low phytic acid and fatty acid alleles from CX1834-1-2. This close linkage could be a problem if a breeder were attempting to combine a high oleic acid allele with the low phytic acid allele in the same genetic background.

Other Physiological Effects

Oltmans et al. (2005) reported that seedling emergence of low phytic acid lines was 30% lower than that of normal phytic acid lines. Advanced backcrossing of low phytic acid lines with high yielding normal lines has resulted in low seed phytic acid lines with normal seedling emergence and vigor (R. Boerma, personal communication, 2006). This indicates that low seedling vigor and germination are not due to the low seed phytic acid trait per se. If there are genetic linkages, these can be broken with breeding methods such as back crossing.

Mutations that disrupted inositol polyphosphate kinases, [I(1,3,4,6)P4]/[I(1,3,4,5,6)P5]2-kinase (Ipk1) and [I(1,3,4,6)P4] 5-kinase (Ipk2), in the lipid-dependent biosynthetic pathway of *Arabidopsis thaliana* L. resulted in a >90% decrease in seed phytic acid P and a 10-fold increase in inorganic P compared to the wild-type (Stevenson-Paulik et al., 2005). They also demonstrated that elimination of Ipk1 activity caused leaf epinasty, decreased leaflet size, and abnormal root development when plants were grown with high P levels (1 mmol L^{-1}). These symptoms were not evident when plants were supplied solutions containing 0.1 mmol L^{-1} P. These results suggest that low phytic acid *Arabidopsis* plants defective in Ipk1 have a problem regulating phosphate uptake when a high concentration is available in the nutrient solution. The impact of the low seed phytic acid trait on physiological processes in leaves stems and roots was not evaluated in the present study. Since plant breeders are having success developing low seed phytic acid lines with acceptable seed yields, impairment of processes in vegetative organs does not appear to be serious problem in these lines. However, since a number of different mutations have been shown to lower seed phytic acid concentrations in crop plants (Hitz et al., 2002; Raboy et al., 2000; Shi et al., 2003, 2005; Wilcox et al., 2000), it would be appropriate to assess the impact of the trait on processes in vegetative organs in future experiments.

CONCLUSIONS

Soybean lines homozygous recessive at one of two loci that condition the low seed phytic acid phenotype (*pha1pha1Pha2Pha2* or *Pha1Pha1pha2pha2*) exhibited the same response to increasing external P supply (greater than threefold increase in phytic acid P) as the normal phytic acid parental line (*Pha1Pha1Pha2Pha2*). This supports conclusions from inheritance and mapping studies that

the low seed phytic acid phenotype is controlled by two recessive genes. The low seed phytic acid phenotype of a line derived from CX1834-1-2 (G03PHY-443) was stable from deficient to excessive levels of external P supply (Fig. 1, 3). G03PHY-443 plants supplied with 0.5 or 0.9 to 1.2 mmol L^{-1} external P levels had seed P compositions similar to those of low phytic acid lines tested in three field environments in Iowa (Oltmans et al., 2005). These results suggest that the low phytic acid phenotype should be expressed when these lines are grown in soils with a wide range of P availabilities.

Increasing the external P supply decreased total protein concentrations and increased oil concentrations in seed of both normal and low seed phytic acid lines. However, these parameters did not differ significantly between normal and low phytic acid lines (Tables 5 and 6). Thus, although seed of the low phytic acid line had much higher inorganic P concentrations than seed of the normal lines, the balance between protein and oil synthesis was not altered. Small changes in fatty acid composition between low phytic acid and normal lines were noted (Tables 7 and 8).

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Table 7. Influence of genotype and external P concentration on fatty acid composition of soybean seed (Experiment 1).

Genotype	Nutrient solution P	Fatty acid methyl esters				
		16:0	18:0	18:1	18:2	18:3
	mmol L^{-1}	Weight %				
G03PHY-443	0.1	12.3	2.7	23.2	53.4	8.4
	0.5	12.7	3.0	30.9	44.6	8.7
	1.2	13.7	3.7	21.7	50.6	10.5
Prichard-RR	0.1	12.1	2.8	21.7	53.8	9.5
	0.5	12.4	2.9	26.0	48.7	9.9
	1.2	12.3	2.9	24.9	49.7.	10.1
	$LSD_{0.05}$	0.4	0.3	2.0	1.2	0.6
		P treatment means				
	0.1	12.2	2.8	22.4	53.6	8.9
	0.5	12.6	3.0	28.4	46.6	9.3
	1.2	13.0	3.3	23.3	50.2	10.3
	$LSD_{0.05}$	0.3	0.2	1.4	0.8	0.4
		Genotypic means				
G03PHY-443		12.9	3.1	25.3	49.5	9.2
Prichard-RR		12.3	2.9	24.2	50.7	9.7
$LSD_{0.05}$		0.3	0.2	NS†	0.7	0.3

†NS, nonsignificant.

Table 8. Influence of low phytic acid trait and P supply on fatty acid composition of soybean oil (Experiment 2).

Genotype†	Nutrient solution P conc.	Fatty acid methyl esters				
		16:0	18:0	18:1	18:2	18:3
	mmol L^{-1}	Weight %				
G03PHY-443	0.05	14.4	3.1	17.4	55.5	9.7
	0.50	15.6	3.6	17.2	53.6	10.0
	0.90	15.4	3.9	17.6	52.3	11.0
Prichard-RR	0.05	13.6	3.4	19.3	53.6	10.1
	0.50	14.2	3.6	19.1	51.2	11.9
	0.90	12.8	3.6	18.9	52.9	11.8
LG L	0.05	12.6	3.5	19.0	54.4	10.5
	0.50	13.1	3.5	18.8	53.6	10.9
	0.90	13.4	3.5	19.0	53.5	10.6
LG N	0.05	13.6	3.3	19.0	54.2	9.8
	0.50	14.0	3.5	19.4	51.2	11.3
	0.90	14.1	3.7	19.3	51.9	11.1
	$LSD_{0.05}$	NS‡	0.2	NS	NS	0.6
		P treatment means				
	0.05	13.6	3.3	18.7	54.4	10.0
	0.50	14.2	3.6	18.6	52.4	11.0
	0.90	13.9	3.7	18.7	52.6	11.1
	$LSD_{0.05}$	0.5	0.1	NS	0.9	0.3
		Genotypic means				
G03PHY-443		15.1	3.5	17.4	53.8	10.2
Prichard-RR		13.5	3.5	19.1	52.6	11.3
LG L		13.0	3.5	18.9	53.8	10.7
LG N		13.9	3.5	19.2	52.4	10.7
$LSD_{0.05}$		0.6	NS	0.7	1.0	0.4

†LG L, linkage group L; LG N, linkage group N.

‡NS, nonsignificant.

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